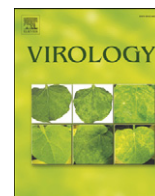




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## Redundancy in the immune system restricts the spread of HSV-1 in the central nervous system (CNS) of C57BL/6 mice

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### ABSTRACT

Resistance to lethal encephalitis in mice infected with HSV-1 via the oral mucosa is mouse strain dependent. In susceptible BALB/c, HSV-1 spreads throughout the CNS but in resistant BL/6 mice, virus is restricted to the brainstem. To examine the contribution of cellular immunity in restricting viral spread, we used a combination of antibody depleted and KO mice. Individually, NK/NKT, iNKT, CD4<sup>+</sup>, CD8<sup>+</sup>, and  $\gamma\delta$  T-cells do not restrict HSV-1 spread. In contrast, virus spreads throughout the CNS of BL/6 CL I KO mice and BL/6 mice treated with either anti-asialoGM1 Ab or both anti-CD8 and anti-NK1.1 mAbs. The results highlight the importance of redundancy in the immune system in restricting viral spread in the CNS, argue for a role of NK/NKT and CD8<sup>+</sup> T-cells in mediating the restriction, and provide a hierarchical order of the individual elements in controlling virus in BL/6 mice infected with HSV-1 via the oral mucosa.

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### Introduction

HSV-1 can infect the skin or mucosa of both humans and experimental animals where it undergoes local replication (Roizman and Knipe, 2001). After entering sensory nerve endings, virus spreads via retrograde axonal transport to the ganglia of the peripheral nervous system (PNS) where a productive infection of neurons ensues (Whitley, 2001). Although infectious virus is eventually cleared from all sites, a latent infection of the PNS ganglia is established in a number of neurons (Gilden et al., 2001; Stevens and Cook, 1971).

In contrast, HSV-1 infection of the CNS is less well understood. In humans, virus is transmitted across synapses and latent virus is reportedly widespread in the CNS (Baringer and Pisani, 1994; Itzakhi et al., 1997). HSV-1 is also the commonest cause of fatal sporadic viral encephalitis in man (Roos, 1999; Whitley et al., 1998) with mortality rates reaching 20 to 30% despite treatment (Kennedy, 2005). In experimental animals, HSV-1 can also cause latent infection of the CNS (Rock and Fraser, 1983) and fatal encephalitis (Whitley, 2001). Natural resistance to mortality following intraperitoneal (i.p.)

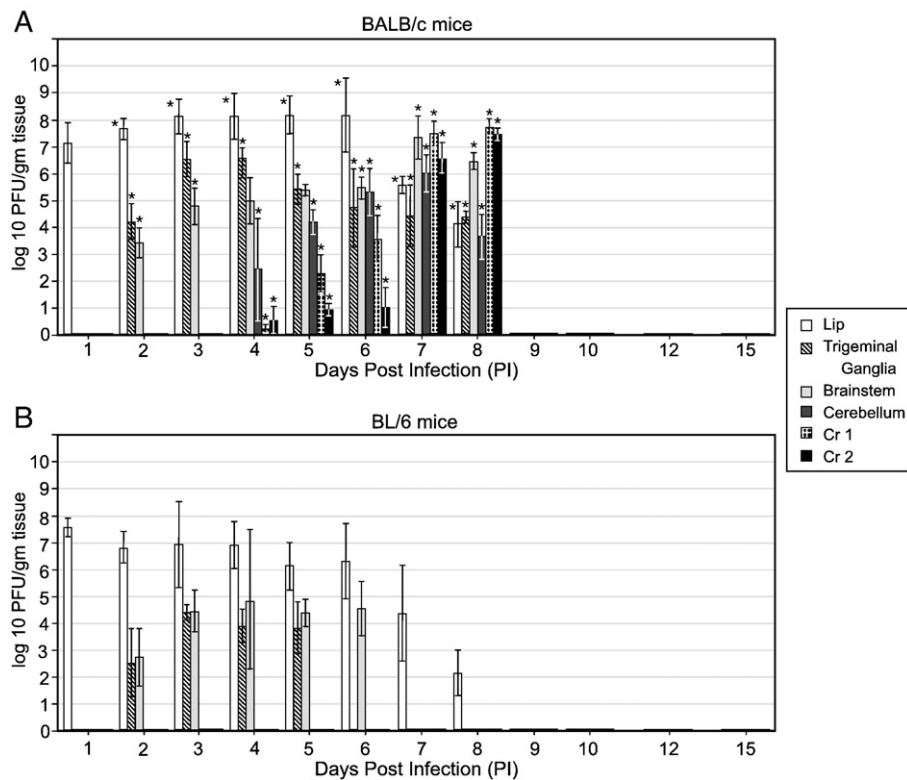
challenge with HSV-1 is a dominant autosomal trait and dependent on the inbred mouse strain (Lopez, 1975). Although, resistance to fatal HSV-1 encephalitis is genetically complex and involves multiple interacting loci (Lundberg et al., 2008), the herpes resistance locus (Hrl) on mouse chromosome 6 has been identified as one determinant of resistance (Lundberg et al., 2003) with TNF playing an important role in mediating resistance (Lundberg et al., 2007). In mice inoculated with HSV-1 via the oral mucosa, natural resistance is also a dominant autosomal trait and mouse strain dependent (Kastrukoff et al., 1986). In this study we used a combination of antibody depleted and KO mice to examine the contribution of cellular immunity in mediating resistance to HSV-1 encephalitis.

### Results

*HSV-1 spreads throughout the CNS in susceptible BALB/c but is restricted to the brainstem in resistant BL/6 mice*

To determine the extent of HSV-1 involvement of the CNS of susceptible BALB/c and resistant BL/6 mice, 10–12 week ♀ mice were infected with a sub-lethal dose of virus via the oral mucosa (Kastrukoff et al., 1986). Viral titers were determined in the lip, trigeminal ganglia (TG), and throughout the brain for up to 15 days PI (Fig. 1). HSV-1 spreads throughout the brain in BALB/c mice

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**Fig. 1.** Analysis of virus titers throughout the brains of 10–12 week ♀ BALB/c (A) and BL/6 (B) mice infected with a sub-lethal dose of HSV-1 via the oral mucosa. Each figure represents results from five independent experiments; no. = 10 mice/time point. \*,  $P < 0.05$  by unpaired two-tailed  $t$  test. Error bars represent SEM.

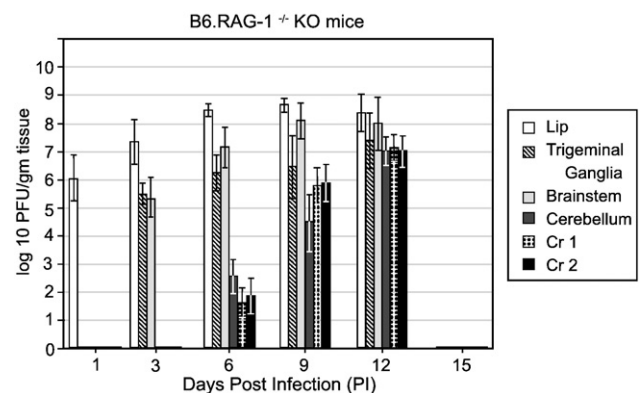
(Fig. 1A) but is restricted to the brainstem (BST) in BL/6 mice (Fig. 1B) before being cleared in both mouse strains. Viral titers were similar in the lip of both strains on day 1 PI but then increased in BALB/c compared to BL/6 mice (~10-fold) by day 3 PI. In the TG, viral titers peaked in both mouse strains on day 3 PI but were significantly greater in BALB/c mice (~100-fold). In the BST, viral titers in BALB/c mice peaked on day 7 PI and again were significantly greater than in BL/6 mice. Infectious virus was first identified in the cerebellum/ cerebrum (CB/CR) of BALB/c mice on day 4/5 PI and peaked on day 7/8 PI (Fig. 1A). Although infectious virus was cleared from all sites by day 9 PI, clearance was delayed in the TG and BST of BALB/c compared to BL/6 mice. The reduced viral load in the TG and BST of BL/6 compared to BALB/c mice, along with restricted viral spread in the brain correlates with the natural resistance to mortality of this strain, following infection of the oral mucosa with HSV-1 (Kastrukoff et al., 1986).

#### HSV-1 spreads throughout the CNS of BL/6 RAG-1 KO (B6.RAG-1<sup>-/-</sup>) mice

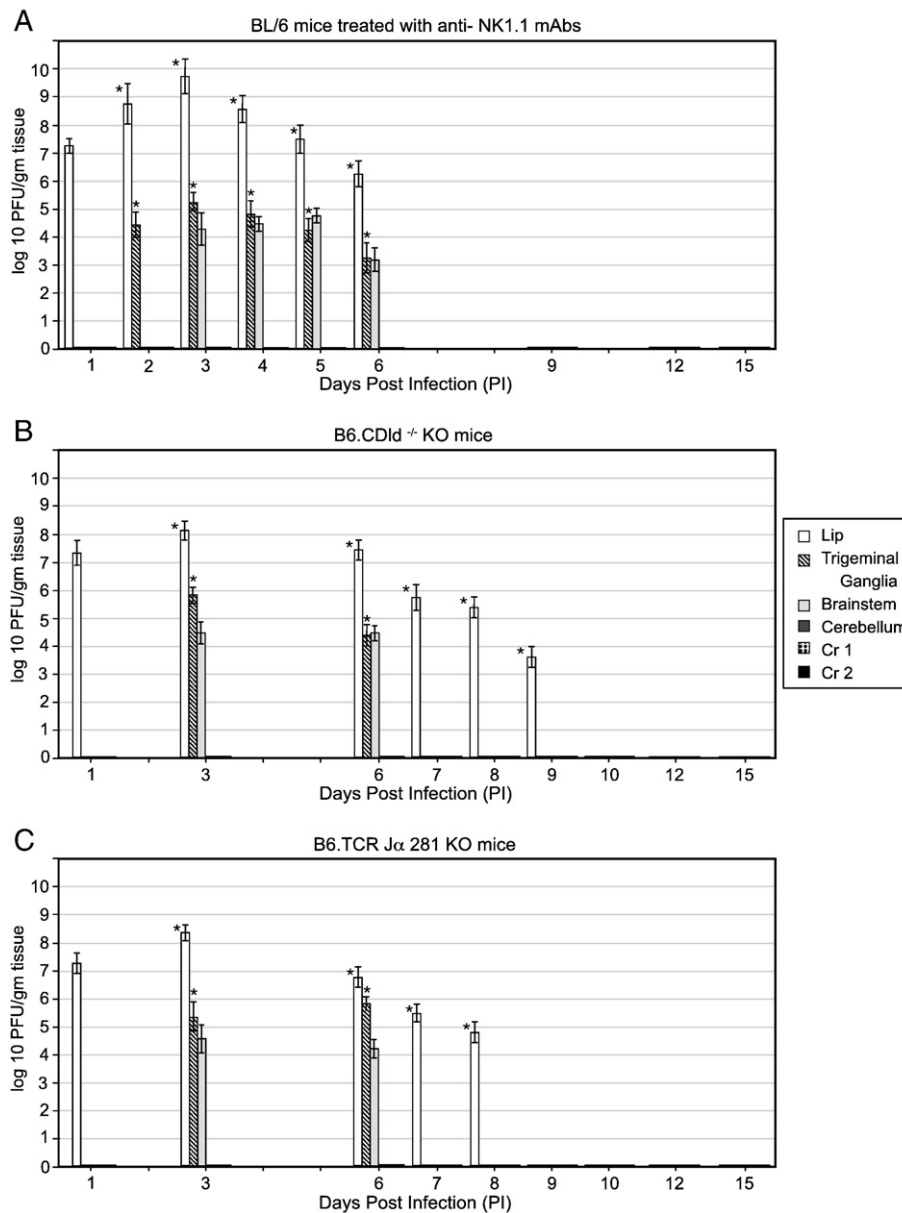
To determine if the immune system contributes to the restriction of HSV-1 spread in the CNS of BL/6 mice, 10–12 week ♀ B6.129S7-Rag1<sup>tm1Mom</sup>/J (B6.RAG-1<sup>-/-</sup> KO) mice (Mombaerts et al., 1992) were infected with a sub-lethal dose of virus via the oral mucosa. These mice are reported to be ten times more susceptible to infection with HSV-1 than BL/6 mice (Vollstedt et al., 2004). Viral titers were determined in the lip, TG, and throughout the brain for up to 15 days PI. HSV-1 spreads throughout the brain of B6.RAG-1<sup>-/-</sup> KO mice (Fig. 2). Viral titers in the lip, TG, and throughout the brain increased steadily and virus was not cleared. Mice became ill by day 9 PI and all succumbed to the infection by day 12 PI. The results support a role for the immune system not only in restricting the spread but also in clearing HSV-1 from the brains of BL/6 mice.

#### NK/NKT cells do not restrict the spread of HSV-1 in the CNS but do restrict viral replication in the oral mucosa of BL/6 mice during early stages of infection

The importance of natural killer (NK) cells in HSV-1 infection remains controversial (Pereira et al., 2001). To determine if they contribute to restricting viral spread in the CNS, ten to twelve week ♀ BL/6 mice received a total of 600 µg of PK136 mAb i.p. and a sub-lethal dose of HSV-1 via the oral mucosa. Viral titers were determined in the lip, TG, and throughout the brain up to 15 days PI (Fig. 3A). CD3<sup>+</sup>NK1.1<sup>+</sup> cells in the spleen were reduced by 85% on day 6 and by 84% on day 15 PI as determined by flow cytometry (data not shown). Comparable levels of depletion with PK136 mAb are reported by other investigators (Postol



**Fig. 2.** Analysis of virus titers throughout the brains of 10–12 week ♀ B6.129S7-Rag1<sup>tm1Mom</sup>/J (B6.RAG-1<sup>-/-</sup> KO) mice inoculated with a sub-lethal dose of HSV-1 via the oral mucosa. The figure represents the results of five independent experiments; no. = 10 mice/time point. Error bars represent SEM.



**Fig. 3.** Analysis of virus titers throughout the brains of 10–12 week BL/6 mice treated with anti-NK1.1 mAb (A) and inoculated with a sub-lethal dose of HSV-1 via the oral mucosa. Viral titers throughout the brains of 10–12 week HSV-1 infected C57BL/6 CD1d-deficient (B6.CD1d<sup>-/-</sup> KO) (B) and C57BL/6 NKT cell-deficient (B6.TCR Jα281 KO) (C) mice were also determined. All figures represent the results of five independent experiments; no. = 10 mice/time point. \*,  $P < 0.05$  by unpaired two-tailed  $t$  test. Error bars represent SEM.

et al., 2008). Although CD3<sup>+</sup>NK1.1<sup>+</sup> cells could not be identified in the BST of PK136 mAb treated mice by flow cytometry (data not shown), the total absence of these cells is difficult to confirm. A number of factors contribute to this including: the small numbers of inflammatory cells in the BST even prior to depletion, the viscosity of the homogenate which interferes with the isolation of cells, the autofluorescence of CNS cells which interferes with flow analysis, and receptor blockade resulting from the pre-treatment of cells with anti-NK1.1 mAb. Despite depletion of CD3<sup>+</sup>NK1.1<sup>+</sup> cells, the spread of HSV-1 in the brains of BL/6 mice remained restricted to the BST.

In the lip, viral titers were similar in both mice treated with PK136 mAb and controls on day 1 PI but then increased significantly in the treated group with titers peaking on day 3 PI (~1000-fold increase). In the TG, viral titers were also increased in the treated group on day 3 PI (~100-fold) while in the BST, viral titers were not significantly different from controls. Virus was cleared from all sites by day 9 PI.

The role of invariant natural killer T (iNKT) cells in HSV-1 infection also remains controversial (Grubor-Bauk et al., 2003; Cornish et al.,

2006). To determine if they contribute to the restriction of viral spread in the CNS, ten to twelve week ♀ BL/6 CD1d-deficient (B6.CD1d<sup>-/-</sup> KO) (Mendiratta et al., 1997) and BL/6 NKT cell-deficient (B6.TCR Jα281 KO) (Cui et al., 1997) mice were infected with a sub-lethal dose of HSV-1 via the oral mucosa. Viral titers in the lip, TG, and throughout the brain were determined up to 15 days PI in B6.CD1d<sup>-/-</sup> KO mice (Fig. 3B) and B6.TCR Jα281 KO mice (Fig. 3C). The spread of HSV-1 in the brains of these mice remained restricted to the BST.

In the lip, viral titers were similar in CD1d-deficient, NKT cell-deficient and control mice on day 1 PI but increased modestly in the deficient groups thereafter. Viral titers were ~10 fold greater in the lip and TG of deficient mice compared to controls on day 3 PI. In the BST, viral titers were not significantly different in deficient mice and controls. Infectious virus was cleared from all sites by day 10 PI.

The results do not support a role for NK/NKT or iNKT cells in restricting the spread of HSV-1 in the CNS of BL/6 mice. In contrast, NK/NKT cells do contribute to the restriction of viral replication in the lip and TG while iNKT cells may have a modest effect at these sites.

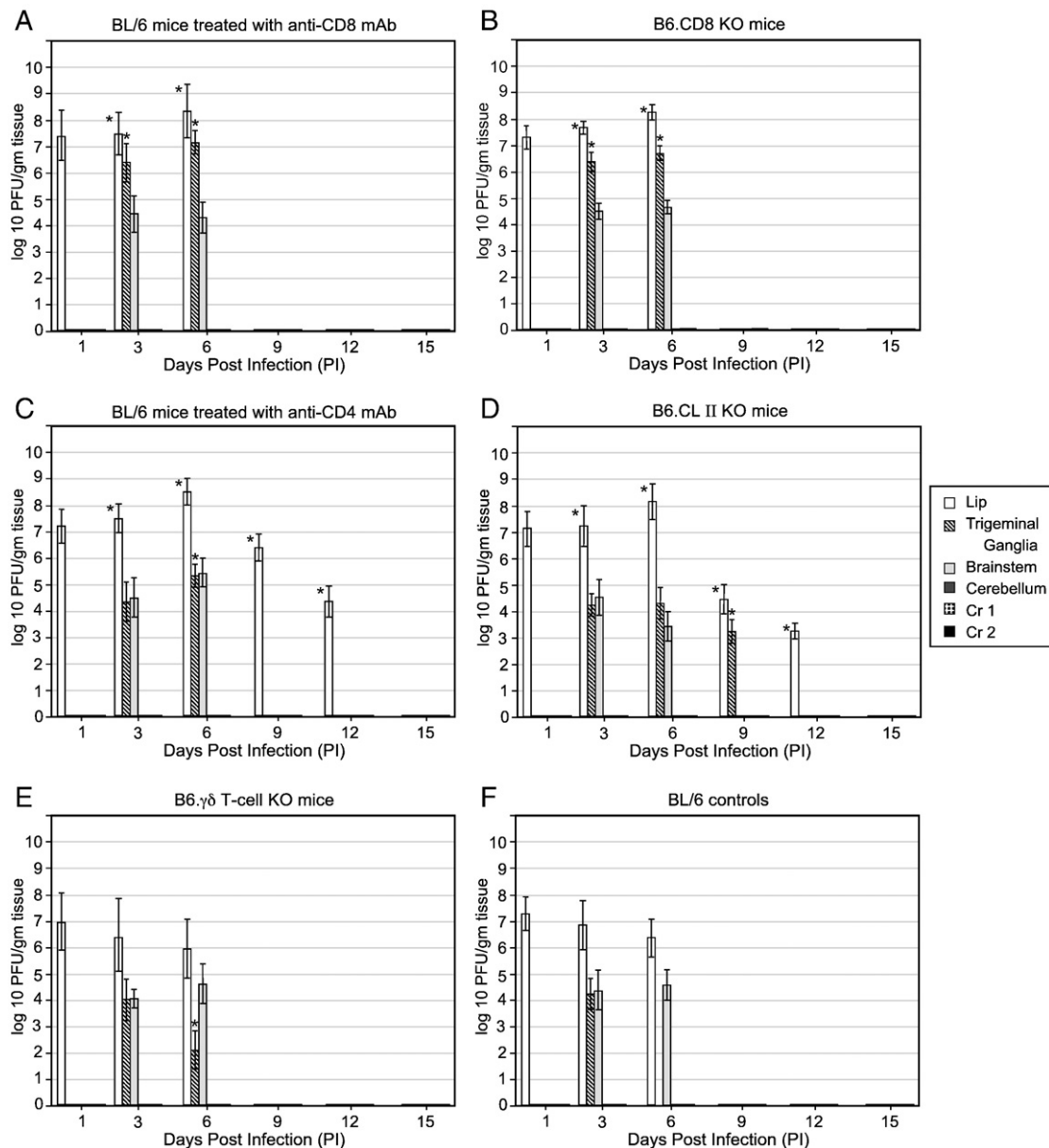
*CD8<sup>+</sup> T-cells do not restrict the spread of HSV-1 in the CNS but do restrict viral replication in the oral mucosa and peripheral ganglia of BL/6 mice during later stages of infection*

CD8<sup>+</sup> T-cells respond to HSV infection by cytokine production or by direct killing of target cells (Bonneau and Jennings, 1989; Larsen et al., 1983, 1984; Nash et al., 1987; Smith et al., 1994; Trapani and Smyth, 2002). As protection against encephalitis has been reported to be the primary domain of CD8<sup>+</sup> T-cells (Newell et al., 1989; Simmons and Nash, 1984; Simmons and Tschärke, 1992), we hypothesized that anti-HSV CD8<sup>+</sup> CTL might also be responsible for restricting the spread of HSV-1 in BL/6 mice. Ten to twelve week ♀ BL/6 mice, depleted of CD8<sup>+</sup> cells, were infected with a sub-lethal dose of HSV-1 via the oral mucosa. Treatment with 2.43 mAb resulted in >95% depletion of CD3<sup>+</sup>CD8<sup>+</sup> spleen cells on days 3, 9, and 15 PI (data not shown). Ten to twelve week ♀ BL/6 CD8 KO (B6.129S2-Cd8a<sup>tm1Mak</sup>/J) (Fung-Leung et al., 1991) mice were also infected with a sub-lethal dose of HSV-1. Viral titers were determined in the lip, TG, and

throughout the brain up to 15 days PI in depleted (Fig. 4A) and KO (Fig. 4B) mice. In both groups of mice, the spread of HSV-1 remained restricted to the BST.

In the lip and TG, viral titers increased in CD8<sup>+</sup> depleted mice on day 3 but peaked by day 6 PI (~100-fold) compared to controls. However, viral titers in the BST were not significantly different in depleted or control mice. Although infectious virus was cleared from all sites by day 9 PI, viral clearance from the TG was delayed compared to controls (Fig. 4F).

Together, results from both depletion studies and KO mice support a role for CD8<sup>+</sup> T-cells in restricting viral replication in the lip and TG but not in restricting the spread of HSV-1 in the brains of BL/6 mice. These results are consistent with those of other investigators (Ghiasi et al., 1999). Although the delay in viral clearance from the TG supports a role for these cells in the clearance of HSV-1, virus is cleared from the lip and TG in both CD8<sup>+</sup> T-cell depleted and KO mice. The results suggest that redundancy in the immune system plays a role in viral clearance at these sites.



**Fig. 4.** Analysis of viral titers throughout the brain of 10–12 week ♀ BL/6 mice depleted of CD8<sup>+</sup> (A) or CD4<sup>+</sup> (C) T-cells and infected with a sub-lethal dose of HSV-1 via the oral mucosa. Ten to twelve week ♀ B6.129S2-Cd8a<sup>tm1Mak</sup>/J (B6.CD8 KO) (B), B6.129-H2<sup>d1Ab1-Ea</sup>/J (B6.CL II KO) (D), B6.129P2-Tcrd<sup>tm1Mom</sup>/J (B6.γδ T-cell KO) (E), and BL/6 (F) mice were also infected with a sub-lethal dose of virus. Each figure represents the results of five independent experiments; no. = 10 mice/time point. \*, *P* < 0.05 by unpaired two-tailed *t* test. Error bars represent SEM.



*CD4<sup>+</sup> T-cells do not restrict the spread of HSV-1 in the CNS but do restrict viral replication in the oral mucosa of BL/6 mice during later stages of infection*

CD4<sup>+</sup> T-cells protect experimental animals from HSV infection (Manicken and Rouse, 1995; Manicken et al., 1995a,b). To determine if these cells might restrict the spread of virus in the CNS of BL/6 mice, ten to twelve week ♀ BL/6 mice, depleted of CD4<sup>+</sup> cells, were infected with a sub-lethal dose of HSV-1 via the oral mucosa. Treatment with GK1.5 mAb resulted in >95% depletion of CD3<sup>+</sup>CD4<sup>+</sup> spleen cells on days 3, 9, and 15 PI (data not shown). Ten to twelve week ♀ BL/6 CL II KO (B6.129-H2<sup>d1Ab1-Ea</sup>/J) mice (Madsen et al., 1999) were also infected with a sub-lethal dose of HSV-1. Viral titers were determined in the lip, TG, and throughout the brain for 15 days PI in depleted (Fig. 4C) and KO (Fig. 4D) mice. In both, the spread of HSV-1 remained restricted to the BST.

In the lip, viral titers were similar in CD4 depleted, KO, and control mice on day 1 PI but increased in depleted and KO mice; peaking on day 6 PI (~100-fold increase). However, viral clearance was delayed in the lip and TG of depleted and KO mice compared to controls.

Together, the results from depletion studies and BL/6 CL II KO mice support a role for CD4<sup>+</sup> T-cells in restricting viral replication in the lip along with clearing virus from the lip and TG but not in restricting the spread of HSV-1 in the brain. The results are similar to reports by other investigators (Ghiasi et al., 1999). Although these cells contribute to the clearance of HSV-1, virus is cleared from the lip and TG despite CD4<sup>+</sup> T-cell depletion and in CL II KO mice. The results suggest that redundancy in the immune system plays a role in viral clearance at these sites.

*γδ T-cells do not restrict the spread of HSV-1 in the CNS of BL/6 mice*

As virus specific γδ T-cells are identified in mice infected with HSV-1 (Johnson et al., 1992; Sciammas et al., 1994), we investigated the possibility that these cells restrict the spread of virus in the CNS of BL/6 mice. Ten to twelve week ♀ BL/6 γδ T-cell KO (B6.129P2-Tcrd<sup>tm1Mom</sup>/J) mice (Itoharu et al., 1993) were infected with a sub-lethal dose of HSV-1 via the oral mucosa. Viral titers were determined in the lip, TG, and throughout the brain up to 15 days PI (Fig. 4E). The spread of HSV-1 remains restricted to the BST in these mice.

This subset of immune cells appears to contribute little to restricting viral replication in the lip, TG, or BST. However, they may have a modest effect in the TG as the clearance of infectious virus is delayed in these mice. Similar results were reported by Liu et al. (1996) using a corneal inoculation model in A/J mice while Sciammas et al. (1997) concluded that γδ T-cells could play a role in the TG during acute HSV-1 infection.

*HSV-1 remains restricted to the brainstem in BL/6 mice depleted of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells while infectious virus persists in the oral mucosa, TG, and brainstem*

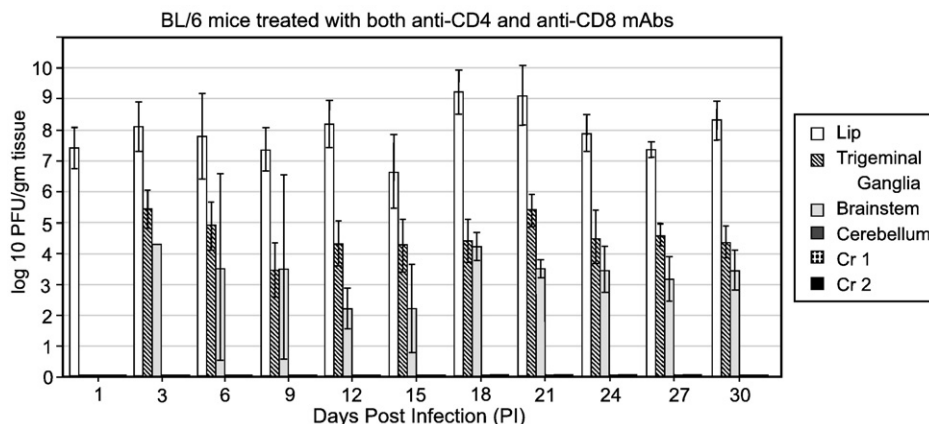
Both CD8<sup>+</sup> and CD4<sup>+</sup> T-cells may be required for optimal protection against HSV infection (Howes et al., 1979; Nash et al., 1981). To determine if both T-cell subsets are required to restrict the spread of HSV-1 in the CNS of BL/6 mice, 10–12 week ♀ BL/6 mice, depleted of CD8<sup>+</sup> and CD4<sup>+</sup> T-cells, were infected with a sub-lethal dose of HSV-1 via the oral mucosa. Treatment with both GK1.5 and 2.43 mAbs resulted in >95% depletion of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> spleen cells on days 3, 9, 15, 21, and 27 PI while neither subset of T-cells was identified in the BST on day 5 or 7 PI (data not shown). Viral titers were determined in the lip, TG, and throughout the brain every three days until day 30 PI (Fig. 5). Despite the depletion of both subsets of T-cells, the spread of HSV-1 in the brains of BL/6 mice remained restricted to the BST.

In the lip, viral titers were increased ~100-fold and in the TG ~10-fold but not in the BST of depleted mice compared with controls. Further, HSV-1 was not cleared and infectious virus persisted in the lip, TG, and BST for at least 30 days PI. Despite this, mice did not become ill or succumb to infection. These results are comparable with earlier reports of viral persistence in the foot-pad for at least 15 days (Smith et al., 1994) and in the TG for at least 35 days (Ghiasi et al., 1999) in CD4<sup>+</sup>/CD8<sup>+</sup> T-cell depleted BL/6 mice. HSV-1 persistence was also reported in the TG of BALB/c mice in the absence of TCRαβ<sup>+</sup> T-cells, but here virus did spread throughout the brain, resulting in lethal encephalitis (Sciammas et al., 1997).

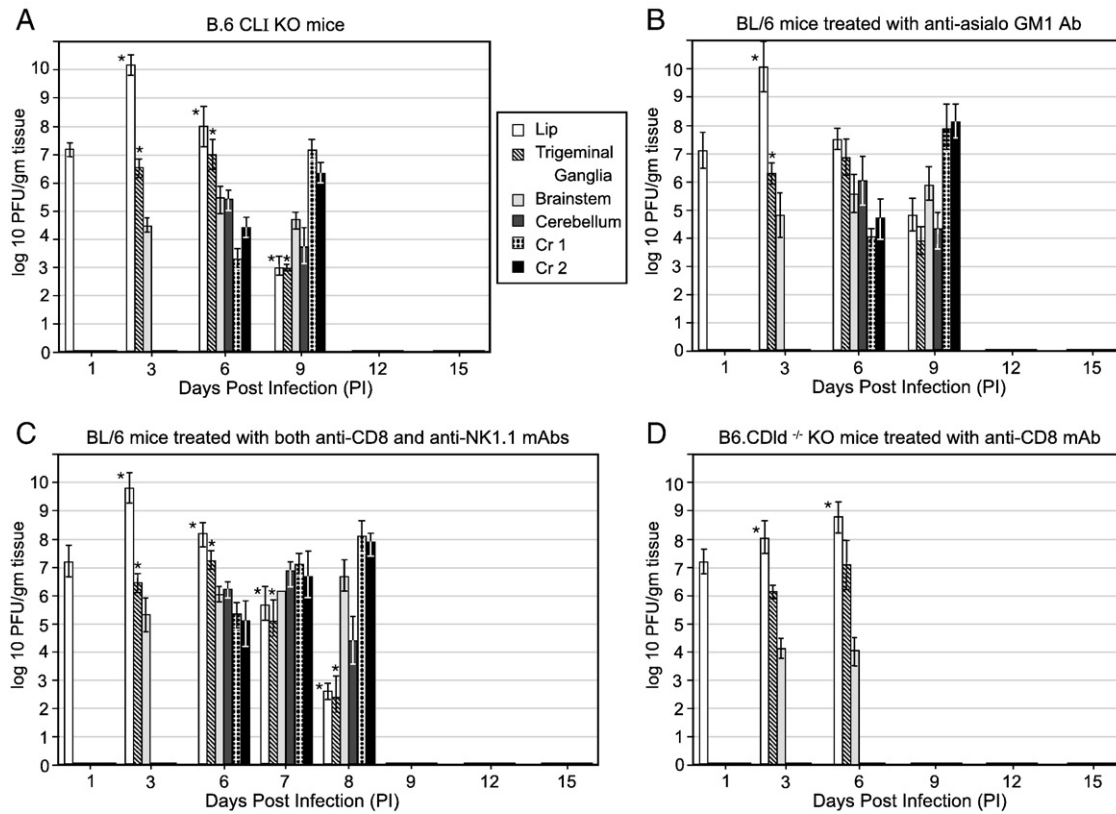
Together the results indicate that redundancy in the immune system plays a role in the clearance of virus from the lip, TG, and BST and is mediated by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. Despite this, HSV-1 remained restricted to the BST of BL/6 mice suggesting that other immune mechanisms are important in restricting the spread of virus in the brain.

*HSV-1 spreads throughout the CNS of BL/6 CL I KO mice, BL/6 mice treated with anti-asialoGM1 Ab, and BL/6 mice treated with both anti-CD8 and anti-NK1.1 mAbs, but not in B6.CD1d<sup>-/-</sup> KO mice treated with anti-CD8 mAb*

Initially, CD8 depleted BL/6 mice and BL/6 CL I KO mice were infected with a sub-lethal dose of virus to determine if CD8<sup>+</sup> T-cells restrict the spread of HSV-1 in the CNS. Although virus remained restricted to the BST in CD8 depleted BL/6 mice, surprisingly, HSV-1 spread throughout the brains of ten to twelve week ♀ B6.CL I KO (B6.129P2-β2m<sup>tm1Unc</sup>/J) mice before being cleared (Fig. 6A). This



**Fig. 5.** Analysis of viral titers throughout the brain of 10–12 week ♀ BL/6 mice treated with both anti-CD8 and anti-CD4 mAb and infected with a sub-lethal dose of HSV-1 via the oral mucosa. The figure represents the results of five independent experiments; no. = 10 mice/time point. Error bars represent SEM.



**Fig. 6.** Analysis of viral titers throughout the brain of 10–12 week ♀ B6.129P2-B2m<sup>tm1unc</sup>/J (B6.CLI KO) (A), BL/6 mice treated with anti-asialoGM1 Ab (B), BL/6 mice treated with both anti-CD8 and anti-NK1.1 mAbs (C), and BL/6 CD1d-deficient (B6.CD1d<sup>-/-</sup> KO) mice treated with anti-CD8 mAb (D) and infected with a sub-lethal dose of HSV-1 via the oral mucosa. The figure represents the results of five independent experiments; no. = 10 mice/time point. \*,  $P < 0.05$  by unpaired two-tailed  $t$  test. Error bars represent SEM.

raised the possibility that redundancy in the immune system might also play a role in restricting the spread of HSV-1 in the CNS. In the KO mice, viral titers in the lip were similar to controls on day 1 PI but increased significantly by day 3 PI (~1000-fold). Viral titers also increased in the TG by day 3 (~100-fold) and 6 PI as well as in the BST. HSV-1 was first identified in the CB/CR after day 3 PI; with titers peaking by day 6/9 PI. Although infectious virus was cleared from all sites by day 12 PI, it was delayed in the lip, TG, and BST compared to controls.

To further investigate the possibility of redundancy, BL/6 mice were treated with anti-asialoGM1 Ab prior to being infected with a sub-lethal dose of HSV-1 via the oral mucosa. In ten to twelve week ♀ BL/6 mice, given a total of 120  $\mu$ l of Ab, viral titers were determined in the lip, TG, and throughout the brain until day 15 PI (Fig. 6B). Viral titers in the lip were similar to controls on day 1 PI but increased significantly by day 3 PI (~1000-fold). Viral titers increased in the TG by day 3 (~100-fold) and 6 PI. In the BST, titers were maximal on day 9 PI. HSV-1 was first identified in the CB/CR after day 3 PI with titers peaking by day 6/9 PI. Although infectious virus was cleared from all sites by day 12 PI, it was delayed in the lip, TG, and BST compared to controls.

BL/6 mice were then treated with anti-CD8<sup>+</sup> and anti-NK1.1 mAbs prior to being infected with a sub-lethal dose of HSV-1 via the oral mucosa. Ten to 12 week ♀ BL/6 mice, given a total of 600  $\mu$ g of PK136 and 600  $\mu$ g of 2.43 mAbs, were infected with a sub-lethal dose of HSV-1. The reduction of CD3<sup>+</sup>NK1.1<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> cells in the spleen was similar to that of single depletion studies and neither population of cells was identified in the BST on day 5 or 7 PI (data not shown). Viral titers were determined in the lip, TG, and throughout the brain until day 15 PI (Fig. 6C). Viral titers in the lip were similar to controls on day 1 PI but increased significantly by day 3 PI (~1000-fold). Viral titers also increased in the TG on day 3 (~100-fold) and 6

PI. In the BST, titers peaked by day 8 PI. HSV-1 was first identified in the CB/CR after day 3 PI and peaked by day 7/8 PI. Although infectious virus was cleared from all sites by day 9 PI, it was delayed in the lip, TG, and BST compared to controls.

In ten to 12 week ♀ BL/6 CD1d-deficient (B6.CD1d<sup>-/-</sup> KO) mice, given a total of 600  $\mu$ g of 2.43 mAb, viral titers were determined in the lip, TG, and throughout the brain until day 15 PI (Fig. 6D). The spread of virus remained restricted to the BST. Viral titers in the lip were similar to controls on day 1 PI but increased by days 3 (~10-fold) and 6 (~100-fold) PI. Viral titers were also increased in the TG by days 3 (~100-fold) and 6 PI. In the BST, viral titers were not significantly different from controls.

Together the results indicate that redundancy in the immune system is important in restricting the spread of HSV-1 in the CNS of BL/6 mice. Further, our results argue that it is mediated by CD8<sup>+</sup> T-cells and NK/NKT cells but not iNKT cells.

## Discussion

Herpes simplex virus type 1 (HSV-1) is a ubiquitous human pathogen (Whitley et al., 1998) that can induce acute and latent infections of the peripheral and central nervous systems (PNS and CNS) (Gilden et al., 2001; Baringer and Pisani, 1994), and cause a number of serious clinical syndromes including neonatal herpetic infection and HSV-1 encephalitis (Whitley, 2001; Arduino and Porter, 2008). In response to HSV-1 infection, a complex immune cascade is triggered that involves both the innate and adaptive immune systems (Nash and Cambouropoulos, 1993; Borysiewicz and Sissons, 1994; Mossman, 2002; Khanna et al., 2004). Although HSV-1 infection in experimental animals has increased our knowledge of the immune response at the primary site of infection and in the PNS (Roizman and Knipe, 2001; Bloom, 2006; Pack and Rouse, 2006), the immune

response to CNS infection is less well understood. Although the objective of this study was to identify the role of cellular immunity in mediating resistance to HSV-1 encephalitis following infection of the oral mucosa, the results are also relevant to the immune response to viral replication and clearance at the primary site of infection and in the PNS.

Following infection of the oral mucosa with a sub-lethal dose of HSV-1, virus reaches the TG of susceptible BALB/c and resistant BL/6 mice simultaneously but titers are significantly increased in the susceptible strain. The results are consistent with those of Simmons (1989) who reported viral titers in peripheral ganglia to be mouse strain dependent but contrast with those of Simmons and LaVista (1989) who reported a delay in virus reaching the peripheral ganglia in resistant strains. Similarly, infection of the oral mucosa with HSV-1 results in a significant increase in viral titers in the BST of BALB/c compared to BL/6 mice. This is in contrast to the studies of Lundberg et al. (2003, 2007) where, following corneal scarification, significant differences in HSV-1 titers in the BST were not identified in resistant BL/6 compared to susceptible 129S6 mice. Although viral titers in the TG or BST could not be used as a surrogate marker for mortality in their model, differences in viral titers in the TG and BST of BL/6 and BALB/c mice correlate with resistance to mortality in these strains after infection of the oral mucosa (Kastrukoff et al., 1986) and allows viral titers to be used as a surrogate marker in this model.

Cellular immunity can contribute to the restriction of viral replication at the primary site of infection and in the PNS (Table 1). The contribution of NK cells remains controversial with some studies supporting their role (Brandt and Salkowski, 1992; Habu et al., 1984; Lopez et al., 1980, 1983; Pereira et al., 2001; Rager-Zisman et al., 1987) while others do not (Bukowski and Welsh, 1986; Chmielarczyk et al., 1983; Rossol-Voth et al., 1991). Similarly, iNKT cells are reported to play a role by some investigators (Grubor-Bauk et al., 2003) but not others (Cornish et al., 2006). In mice infected with HSV-1 via the oral mucosa, results from depletion studies support a role for NK/NKT cells in restricting viral replication in the lip and TG during early stages of infection. Similar results were obtained by Reading et al. (2006) in BL/6 mice depleted of NK cells and infected intranasally but not by Kassim et al. (2009) in mice infected in the foot-pad. These results raise the possibility that NK/NKT cells may play a role in mucosal but not skin infections with HSV-1. In contrast, our results support only a modest effect of iNKT cells and no effect by  $\gamma\delta$  T-cells in restricting viral replication in the lip and TG. Although both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells are reported to contribute to the control of primary HSV-1 infection (Carr and Tomanek, 2006), it is generally agreed that either T-cell subset can control infection in the periphery while CD8<sup>+</sup> T-cells

control infection in the peripheral ganglia (Pack and Rouse, 2006). In mice infected with HSV-1 via the oral mucosa, results from both depletion studies and KO mice support roles for both subsets of T-cells in restricting viral replication in the lip during later stages of infection. Our results also support the observation that CD4<sup>+</sup> T-cells do not contribute significantly to the restriction of viral replication in the peripheral ganglia but rather this is mediated by CD8<sup>+</sup> T-cells.

Cellular immunity also plays a role in the clearance of infectious HSV-1 from the lip, TG, and BST after infection of the oral mucosa (Table 1). In both HSV-1 infected BALB/c and BL/6 mice, virus is cleared from all sites by day 9 PI. In BL/6 mice, the clearance of infectious virus from the lip is similar to that of the foot-pad (Smith et al., 1994) and skin (Simmons and Tscharke, 1992) while clearance from the peripheral ganglia after infection of the oral mucosa, is similar to that following other routes of inoculation (Bonneau and Jennings, 1989; Cantin et al., 1999a,b; Liu et al., 1996, 2000; Simmons and Tscharke, 1992; Smith et al., 1994). Individually, NK/NKT, iNKT,  $\gamma\delta$  T-cells, CD4<sup>+</sup>, and CD8<sup>+</sup> T-cells are not essential for the clearance of infectious virus from the lip, TG, or BST as virus is cleared despite the depletion of these immune cell subsets. However, the depletion of these subsets can affect the kinetics of viral clearance. There is a delay in the clearance of virus from the lip and TG of CD4<sup>+</sup> T-cell depleted BL/6 and BL/6 CL II KO mice. Although previously reported, the delay was unexplained (Ghiasi et al., 1999). One possible explanation involves the lack of CD4<sup>+</sup> T-cell help to effector CD8<sup>+</sup> CTL and B-cells (Iijima et al., 2008). While many viruses generate CD8<sup>+</sup> CTL in the absence of helper cells (Mintern et al., 2002; Smith et al., 2004), the need for CD4<sup>+</sup> T-cell help in the development of anti-HSV CD8<sup>+</sup> CTL remains unclear (Behrens et al., 2004). The need for T-cell help has been reported by some (Jennings et al., 1991; Smith et al., 2004) but not others (Vasilakos and Michael, 1993) while still others suggest that help may only be required by specific mouse strains (Mercadal et al., 1991). Recently, the lack of CD4<sup>+</sup> T-cells was shown to be associated with a partial rescue of dysfunctional CD8<sup>+</sup> CTL by NK cells (Nandakumar et al., 2008) and may explain, in part, the observed delay in viral clearance. Clearance of virus from the TG is also delayed in BL/6  $\gamma\delta$  T-cell KO mice and BL/6 mice depleted of CD8<sup>+</sup> T-cells or BL/6 CD8 KO mice. This is consistent with the time required to activate virus specific CD8<sup>+</sup> T-cells (Coles et al., 2002) and to localize to the site of inflammation (van Lint et al., 2005). A similar effect was observed in zosteriform (van Lint et al., 2004) and corneal (Land and Nikolich-Zugich, 2005) models. Our results do support a role for redundancy in the immune system in clearing HSV-1 from the lip, TG, and BST. Although virus is cleared from all three sites in HSV-1 infected BL/6 mice depleted of either CD4<sup>+</sup> or CD8<sup>+</sup> T-cells, HSV-1 persists when both T-cell subsets are depleted. In these mice, virus persists for at least 30 days PI. Similar results have been reported by some (Ghiasi et al., 1999; Smith et al., 1994) but not all investigators (Reading et al., 2006).

Resistance to HSV-1 encephalitis is genetically complex and involves multiple interacting loci (Lundberg et al., 2008). As a result, it has been difficult to identify mechanisms contributing to resistance although TNF has been determined to play an important role (Lundberg et al., 2007). After infection of the oral mucosa, HSV-1 was identified throughout the brains of mice either dying or near death from viral encephalitis (Kastrukoff et al., 1986, 1993). This led us to hypothesize that lethal HSV-1 encephalitis results from the inability of the host to restrict the spread of virus, to control viral replication, or to clear virus from the CNS. Further, we reasoned that it might be possible to identify mechanisms mediating resistance to encephalitis by using a sub-lethal dose of HSV-1.

This study identifies two mechanisms in different mouse strains that likely contribute to resistance to HSV-1 encephalitis following infection of the oral mucosa. In BL/6 mice, virus is restricted to the BST while in BALB/c mice, HSV-1 spreads throughout the brain but is then cleared without mortality. Support for the second mechanism comes

**Table 1**  
Role of cellular immunity in restricting HSV-1 replication and clearance.

	Oral mucosa	Trigeminal ganglia (PNS)	Brainstem (CNS)
Restriction of viral replication			
NK/NKT cells	+++ <sup>a</sup>	++ <sup>a</sup>	–
iNKT cells	+ <sup>a</sup>	+ <sup>a</sup>	–
$\gamma\delta$ T-cells	–	–	–
CD8 <sup>+</sup> T-cells	++ <sup>b</sup>	++ <sup>b</sup>	–
CD4 <sup>+</sup> T-cells	++ <sup>b</sup>	–	–
CD8 <sup>+</sup> and CD4 <sup>+</sup> T-cells	++ <sup>b</sup>	+	–
Viral clearance			
NK/NKT cells	–	–	–
iNKT cells	–	–	–
$\gamma\delta$ T-cells	–	D	–
CD8 <sup>+</sup> T-cells	–	D	–
CD4 <sup>+</sup> T-cells	D	D	–
CD8 <sup>+</sup> and CD4 <sup>+</sup> T-cells	NC	NC	NC

Depletion results in +++ = 1000 fold; ++ = 100 fold; + = 10 fold increase in viral titers. D = delay in viral clearance; NC = virus is not cleared and persists for >30 days.

<sup>a</sup> Day 3 PI.

<sup>b</sup> Day 6 PI.



from HSV-1 infected B6.RAG-1<sup>-/-</sup> KO mice. In the KO mice, virus spreads throughout the brain with viral titers reaching levels identified in BALB/c mice. However, unlike BALB/c mice, infectious virus in the brains of the KO mice is not cleared and all mice succumb to lethal encephalitis within 12 days PI. Further, results from HSV-1 infected B6.RAG-1<sup>-/-</sup> KO mice indicate that both restriction of viral spread and viral clearance in the brains are immune mediated. These results contrast with those of Lundberg et al. (2003, 2007) where lethal encephalitis correlates with the extent and nature of the inflammation in the BST rather than viral titers (Lundberg et al., 2008). It is likely that different mechanisms mediate resistance to mortality in different models of HSV-1 encephalitis. Further, the results underscore the complexity of mortality as an outcome measure in the different models of lethal HSV-1 encephalitis.

Redundancy in the immune system is recognized to play an important role in a number of viral infections including HSV-1. In this study, we used a combination of antibody depleted and KO mice to examine the role of cellular immunity in restricting the spread of HSV-1 in the brains of BL/6 mice. Results from these studies indicate that CD4<sup>+</sup> and CD8<sup>+</sup> T-cells alone do not restrict the spread of HSV-1. The spread of HSV-1 is also restricted in B6.CD1d<sup>-/-</sup>, B6.TCRJ $\alpha$ 281, and B6.129P2-Tcrd<sup>tm1Mom</sup>/J mice indicating that NKT and  $\gamma\delta$  T-cells are not critical to the restriction of viral spread. Further, HSV-1 remains restricted in BL/6 mice depleted of NK/NKT cells or both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. In contrast, HSV-1 does spread throughout the brain of BL/6 CL I KO mice and raised the possibility that redundancy in the immune system may play a role in restricting viral spread. In  $\beta$ 2m<sup>tm1Unc</sup> mutant mice (Koller et al., 1990), conventional CD8<sup>+</sup> T-cells (Zijlstra et al., 1990) and iNKT cells (Adachi et al., 1995) are significantly depleted. Further, although NK cell numbers are normal (Raulet, 1994) in these mice, their function may be impaired (Raulet and Vance, 2006; Yokoyama and Kim, 2006). Less likely, the spread of virus in the brains of these KO mice could result from a linked flanking gene rather than a CL I effect (Wolfer et al., 2002). To further examine the possibility of redundancy, BL/6 mice were treated with anti-asialoGM1 Ab and infected with HSV-1. Wuest and Carr (2008) reported that BL/6 mice, treated with anti-asialoGM1 Ab and infected with HSV-1 via ocular scarification, were depleted of NK cells and HSV specific CD8<sup>+</sup> T-cells, but not other immune cells. We also observed a similar effect of anti-asialoGM1 Ab on NK cells and CD8<sup>+</sup> T-cells in BL/6 mice infected with HSV-1 via the oral mucosa (Kastrukoff et al., 2004). In these mice, virus is no longer restricted to the BST and spreads throughout the brain before being cleared. Similarly, HSV-1 spreads throughout the brain of infected BL/6 mice treated with both anti-NK1.1 and anti-CD8 mAbs. The results from three independent studies converge and argue that redundancy in the immune system, mediated by NK/NKT and CD8<sup>+</sup> T-cells, contributes to the restriction of HSV-1 spread in the brains of BL/6 mice. The failure of HSV-1 to spread throughout the brains of B6.CD1d<sup>-/-</sup> KO mice treated with anti-CD8 mAb makes it unlikely that iNKT cells contribute to the restriction of HSV-1 spread in the brains of BL/6 mice but cannot entirely exclude a role for other NKT cell such as mNKT (Treiner et al., 2003). Support for NK cells and CD8<sup>+</sup> T-cells being responsible for restricting the spread of HSV-1 in the brain comes from the studies of Wuest and Carr (2008). Using flow cytometric analysis, they identified, among others, the presence of NK cells and CD8<sup>+</sup> T-cells, but not NKT cells in the TG and BST of BL/6 mice infected with HSV-1 via the trigeminal nerve. Results from our flow cytometric studies confirm their observations.

## Materials and methods

### Mice

Eight to ten week old  $\varnothing$  C57BL/6, BALB/c, B6.129S7-Rag1<sup>tm1Mom</sup>/J (B6. RAG-1<sup>-/-</sup> KO) (10th generation backcross to BL/6), B6.129P2-Tcrd<sup>tm1Mom</sup>/J (12th generation backcross to BL/6), B6.129S2-Cd8a<sup>tm1Mak</sup>/J (B6.CD8 KO) (13th generation backcross to BL/6), B6.129-H2d<sup>1Ab1-Ea</sup>/J

(B6.CL II KO) (12th generation backcross to BL/6), and B6.129P2-B2m<sup>tm1Unc</sup>/J (B6. CL I KO) (11th generation backcross to BL/6) mice were purchased from Jackson Laboratories and used at ten to twelve weeks of age. Breeding pairs of C57BL/6 CD1d-deficient (B6.CD1d<sup>-/-</sup>) and C57BL/6 NKT cell-deficient (B6.TCR J $\alpha$ 281 KO) mice were kindly provided by Dr. Mark Smyth (Melbourne, Au). Both CD1d-deficient and NKT cell-deficient mice were backcrossed to BL/6 mice for 10 generations. All mice were housed under specific pathogen-free conditions in animal facilities, Faculty of Medicine, University of British Columbia. All investigations followed the guidelines of the Institutional Animal Care and Use Committee of the University of British Columbia.

### Virus and cells

HSV-1 (lab strain 2) was grown on BHK-21 cells and viral titers determined by plaque assay (Kastrukoff et al., 1982). HSV-1, lab strain 2, was originally isolated from human trigeminal ganglia (TG), plaque purified, and characterized by Dr. Moira Brown (Institute for Virology, Glasgow) (Kastrukoff et al., 1981, 1982). Similar to i.p. challenge (Lopez, 1975), natural resistance to mortality following inoculation of the oral mucosa with this strain of HSV-1 is a dominant autosomal trait and dependent on the inbred mouse strain (Kastrukoff et al., 1986). Further, inoculation with a sub-lethal dose of this strain results in the development of virus induced CNS demyelination in susceptible inbred strains of mice (Kastrukoff et al., 1987, 1992). Virus was stored at -80 °C until required. The oral mucosa was inoculated with  $2 \times 10^5$  PFU of virus using a scarification method described previously (Kastrukoff et al., 1986).

### Viral titers

Viral titers of HSV-1 in the lip, TG, BST, CB, and CR were determined as previously described (Kastrukoff et al., 1986). Briefly, mice were sacrificed at specific intervals PI. A craniotomy was performed and incisions made to separate the cervical spinal cord from the brainstem, the midbrain from the cerebral hemispheres, the brainstem from the cerebellum, and the cerebral hemispheres from each other. Tissue was freeze-thawed three times and disrupted with a Ten Broeck homogenizer prior to centrifugation. Serial dilutions of the supernatant were plaque-assayed on CV1 cells as previously described (Kastrukoff et al., 1986).

### Antibodies

Anti-NK1.1 (PK136), anti-CD4 (GK 1.5), and anti-CD8 (2.43) mAb were purchased from the National Cell Culture Center (Minneapolis, MN) and used in cell depletion studies.

### NK/NKT cell depletion using anti-NK1.1 (PK136) mAb

Ten to 12 week old  $\varnothing$  BL/6 mice were treated with anti-NK1.1 mAb (Koo and Peppard, 1984; Koo et al., 1986; Wang et al., 1998). Two hundred  $\mu$ g of PK136 mAb was given i.p. on days -2, 0, and +7. The optimal amount of mAb for the total in-vivo depletion of NK cells on days +1 and +7 had previously been determined by a functional assay using <sup>51</sup>Cr labeled YAC-1 target cells (Kastrukoff et al., 2004) and is consistent with other investigators (Muhlen et al., 2004; Kassim et al., 2009). Control mice received equivalent amounts of purified rat IgG. Viral titers were determined in the lip, TG, BST, CB, and CR in ten mice per day from day 1 through 6 PI as well as days 9, 12, and 15 PI.

### CD4<sup>+</sup> and/or CD8<sup>+</sup> T-cell depletion using anti-CD4 (GK 1.5) and/or anti-CD8 (2.43)

Ten to 12 week old  $\varnothing$  BL/6 mice were treated with either GK1.5, 2.43, or both mAbs (Ghiasi et al., 1999). One hundred  $\mu$ g of either mAb



was given i.p. on days  $-4$ ,  $-1$ ,  $+2$ ,  $+5$ ,  $+11$ , and  $+14$  while  $100\text{ }\mu\text{g}$  each of anti-CD4 and anti-CD8 was given on days  $-4$ ,  $-1$ ,  $+2$ ,  $+5$ ,  $+11$ ,  $+17$ , and  $+23$ . Control mice received equivalent amounts of purified rat IgG.

#### *NK/NKT and CD8<sup>+</sup> T-cell depletion using anti-asialoGM1 Ab*

Ten to 12 week old ♀ BL/6 mice were treated with anti-asialoGM1 Ab (Wako Chemicals) (Smyth et al., 2001). Forty  $\mu\text{l}$  of anti-asialoGM1 Ab was given i.p. on days  $-1$ ,  $0$ , and  $+7$ . The optimal amount of Ab for the total in-vivo depletion of NK cell functional activity on days  $+1$  and  $+7$  had previously been determined using  $^{51}\text{Cr}$  labeled YAC-1 target cells and by phenotypic analysis using flow cytometry. Similarly, the optimal amount of Ab for the in-vivo depletion of gB<sub>498–505</sub> specific CTL in draining cervical lymph nodes on day  $+5$  was determined by a 4 h  $^{51}\text{Cr}$  release assay using EL4 cells pulsed with gB<sub>498–505</sub> as target cells (Kastrukoff et al., 2004). The amount of Ab required is consistent with other investigators (Wuest and Carr, 2008). Control animals received equivalent amounts of purified rat IgG. Viral titers were determined in the lip, TG, BST, CB, and CR in ten mice per day on days 1, 3, 6, 9, 12, and 15 PI.

#### *NK/NKT and CD8<sup>+</sup> T-cell depletion using anti-NK1.1 (PK136) and anti-CD8 (2.43) mAbs*

Ten to 12 week old ♀ BL/6 mice were treated with both anti-NK1.1 and anti-CD8 mAbs. Two hundred  $\mu\text{g}$  of PK136 mAb was given i.p. on days  $-2$ ,  $0$ , and  $+7$  while  $100\text{ }\mu\text{g}$  of 2.43 mAb was given i.p. on days  $-4$ ,  $-1$ ,  $+2$ ,  $+5$ ,  $+11$ , and  $+14$ . Control mice received equivalent amounts of purified rat IgG. Viral titers were determined in the lip, TG, BST, CB, and CR in ten mice per day on days 1, 3, 6, 7, 8, 9, 12 and 15 PI.

#### *CD8<sup>+</sup> T-cell depletion in BL/6 CD1d<sup>-/-</sup> KO mice with anti-CD8 (2.43) mAb*

Ten to twelve week old ♀ BL/6 CD1d-deficient (B6.CD1d<sup>-/-</sup> KO) mice were treated with  $100\text{ }\mu\text{g}$  of 2.43 mAb given i.p. on days  $-4$ ,  $-1$ ,  $+2$ ,  $+5$ ,  $+11$ , and  $+14$  (Chen et al., 1997; Smiley et al., 1997). Control mice received equivalent amounts of purified rat IgG. Viral titers were determined in the lip, TG, BST, CB, and CR in ten mice per day from days 1, 3, 6, 9, 12, and 15 PI.

#### *Flow cytometry*

Surface markers were analyzed using anti-mouse anti-CD3 $\epsilon$  (145-2C11), anti-CD4 (RM4-4), anti-CD8 (C53.6.7), anti-NK1.1 (PK136), and anti-CD45 conjugated with APC, FITC, or PE (BD Pharmingen).

Single cell suspensions of spleen cells from two mice were obtained from day 3 through 27 PI followed by osmotic lysis of erythrocytes using a NH<sub>4</sub>Cl solution (Sigma-Aldrich). Viability counts were performed in trypan blue.

In some studies, TG and BST were obtained and pooled from five mice sacrificed on day 3, 5, or 7 PI. Tissues were disrupted with a Ten Broeck homogenizer (Fischer Scientific) and the suspension passed through a  $70\text{ }\mu\text{m}$  nylon cell strainer.

Samples were blocked with  $2\text{ }\mu\text{l}$  of Fc Block anti-mouse CD16/32 (BD Pharmingen) and incubated for 20 min on ice. Two  $\mu\text{l}$  of rat serum was added for a further incubation of 20 min. Solutions of (1:100 dilution) anti-CD3 $\epsilon$ , anti-CD4, anti-CD8, or anti-NK1.1 were added as well as anti-mouse CD45 and incubated for 30 min on ice. Cells were labeled with anti-CD3 $\epsilon$  and anti-NK1.1, anti-CD3 $\epsilon$  and anti-CD4 (RM4-4), anti-CD3 $\epsilon$  and anti-CD8 (C54.6.7) or anti-CD3 $\epsilon$  and both anti-CD4 and anti-CD8. The RM4-4 clone was previously determined not to cross react with GK1.5 while the C53.6.7 clone was determined not to react with 2.43 (unpublished results). Cells were washed three times with 1.0% BSA in  $1\times$  PBS. The single cell suspensions were fixed

overnight in 1.0% paraformaldehyde, and resuspended in 1.0% BSA in PBS for analysis on a FACSCaliber (BD Biosciences). Analysis of cells obtained from TG and BST followed the method of Wuest and Carr (2008) with events gated by forward and side scatter as well as by high expression of CD45 Ag.

#### *Statistics*

All analyses for statistically significant differences were performed with Student's unpaired two-tailed *t* test.  $P<0.05$  is considered significant. Results are expressed as mean  $\pm$  SEM.

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